Inhibition of Neutrophil Elastase in CF Sputum by L-658,758

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Accepted for publication August 28, 1997

ABSTRACT

Elastases in cystic fibrosis (CF) pulmonary fluids damage lung tissue and perpetuate cycles of infection, inflammation and injury. Elastases from three different sources may be present in CF airways: neutrophils, macrophages and Pseudomonas. We measured how well the cephalosporin-based antielastase L-658,758 blocks the activity of human neutrophil elastase (NE), human proteinase-3, human macrophage metalloelastase, mouse macrophage metalloelastase and Pseudomonas aeruginosa elastase. We also examined the ability of L-658,758 to block elastases in CF sputum in vitro. Sputum samples from adult CF patients were fractionated to obtain the aqueous sol phase. These were then studied individually or pooled. Elastinolytic activity, which ranged from 3.2 μg elastin degraded/ml sol/min to 26.3 μg elastin degraded/ml sol/min, was measurable in every individual sol sample and in the pooled sol. L-658,758 effectively inhibited elastolysis by NE, proteinase-3 and the pooled sol but did not inhibit the activity of the metalloelastases, human and mouse macrophage metalloelastase and Pseudomonas elastase. Secretory leukoprotease inhibitor, which inhibited NE but did not inhibit proteinase-3, blocked 90% of sol elastinolytic activity; this suggests that the majority of this activity in the pooled sol derived from NE. L-658,758 was an effective inhibitor of sol elastase, blocking more than 97% of elastinolytic activity in the individual sol samples. We conclude that L-658,758 is an effective inhibitor of NE, proteinase-3 and CF sputum sol elastase.

CF is characterized by chronic pulmonary infection and inflammation and a progressive loss of pulmonary function (Davis et al., 1996). It is thought that repeated cycles of infection, particularly with Pseudomonas aeruginosa, and subsequent inflammation contribute to CF lung pathology (Berger, 1991). Although the mechanisms by which inflammation induces lung injury are not fully understood, studies of the contents of CF sputum and BAL have identified a variety of neutrophil proteases and bacterial products with the potential to contribute to pulmonary disease.

In particular, high levels of elastinolytic activity have been detected in sputum and BAL from patients with CF (Fick et al., 1984; Jackson et al., 1984; Suter et al., 1984; Bruce et al., 1985; Tournier et al., 1985). Three different potential sources of elastolytic activity are present in the lungs in CF. Neutrophil secretory granules contain large quantities of the elastinolytic serine proteases NE and proteinase-3 (Bieth, 1986); Pseudomonas aeruginosa produces a metalloelastase (Morihara et al., 1965; Wretlind and Pavlovskis, 1983); lung macrophages produce other matrix metalloproteinases capable of degrading elastin, including 92-kD gelatinase, matrilysin and macrophage metalloelastase (Banda and Werb, 1981; Chapman and Stone, 1984; Shapiro et al., 1993).

Many studies have examined the potential of these elastases to damage the lungs and to perpetuate cycles of infection, inflammation and injury. NE, proteinase-3, human macrophage metalloelastase and Pseudomonas elastase are capable of degrading proteins of the lung extracellular matrix in vitro (Bieth, 1986; Hamdaoui et al., 1987; Rao et al., 1991; Shapiro, 1994). In addition, NE, proteinase-3 and Pseudomonas elastase, when instilled directly into rodent lungs, induce injury in many forms, including hemorrhage and emphysema (Kao et al., 1988; Lucey et al., 1988; Williams et al., 1992). Notably, when they are present in excess, both NE and Pseudomonas elastase are capable of degrading the major antiproteases of the lungs, α1-PI and SLPI, thereby reducing the lungs’ primary protection against harmful proteolysis (Baumstark et al., 1977; Morihara et al., 1979; Johnson et al., 1982; Cantin et al., 1989; Suter and Chevallier, 1991). In addition, NE can actively promote inflammation, and thus perpetuate itself in the lungs, by stimulating human epithelial cells to produce IL-8, a potent neutrophil chemoattract-

ABBREVIATIONS: CF, cystic fibrosis; BAL, bronchoalveolar lavage fluid; NE, neutrophil elastase; α1-PI, α1-proteinase inhibitor; SLPI, secretory leukoprotease inhibitor; CMK, methoxysuccinyl-alala-pro-val-chloromethylketone; L-658,758, 3-acetoxymethyl-7-[S]-methoxy-8-oxo-5-thia-1-aza-6(R)-bicyclo-[4.2.0]oct-2-ene-2-(2-(S)-carboxypyrrolidine-carboxamide)-5,5-dioxide; AAPV-pNA, N-methoxysuccinyl-alala-pro-val-p-nitroanilide.

Received for publication October 18, 1996.

1 Supported by NIH HL 31029, NIH HL08672, HL 43510, CFF I555 and Merck Research Laboratories.

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tant (Nakamura et al., 1992; Richman-Eisenstat et al., 1993). In keeping with this, levels of IL-8 in the epithelial lining fluid of CF patients are reduced by treatment with aerosolized rSLPI (McElvaney et al., 1992).

Elastases may also compromise host defenses against infection. Elastases degrade IgG and IgA, as well as the CR1 receptor on neutrophils and C3bi fragments on Pseudomonas, all of which could act to diminish Pseudomonas opsonization and killing (Fick et al., 1984; Tosi et al., 1990). Furthermore, elastases introduced into rodent lungs have been shown to increase adherence of Pseudomonas to tracheal epithelia (Woods et al., 1980; Plotkowski et al., 1989). Exposing epithelium to these elastases leads to increased secretion of mucus (Klinger et al., 1984; Christensen et al., 1987; Somerville et al., 1991), as well as to decreased ciliary beat frequency (Amitani et al., 1991).

All of these effects, if they occurred in vivo, would have serious consequences for CF patients, for whom pulmonary infection and deterioration can be life-threatening. Given the potential for injury demonstrated by elastases, an arsenal of anti-NE drugs is under development for use in disease states characterized by chronic inflammation. Among these are a family of β-lactam anti-elastases, which are mechanism-based, time-dependent inhibitors of NE (Doherty et al., 1986; Davies et al., 1991). Many have been shown to be potent, specific and stable inhibitors of NE activity in vitro (Bonney et al., 1989; Knight et al., 1992a; Knight et al., 1992b).

Our goal in the studies reported here was to determine how well the cephalosporin-based anti-elastase L-658,758 blocks the activity of human neutrophil elastase, human proteinase-3, human macrophage metalloelastase, mouse macrophage metalloelastase and Pseudomonas aeruginosa elastase. In addition, we measured its ability to block elastolysis by CF sputum sol. Finally, we examined the ability of a panel of inhibitors to block the activity of elastases in CF sputum in vitro.

**Materials and Methods**

**CF sputum sol.** Patients for this study were adults with moderate to advanced CF who were hospitalized at Children's Hospital (Boston, MA) for treatment of acute pulmonary exacerbation. Sputum was collected on ice after the patients' morning chest physiotherapy. Within 4 h, it was fractionated in the cold, by centrifugation at 50,000 × g for 90 min, into a gel pellet containing cells and a sol supernatant containing extracellular enzymes (Jackson et al., 1984). Sputum sol was stored at −70°C until analysis.

**Elastases.** Proteinase-3 was prepared from lysates of leukocyte granules obtained from patients with chronic myeloid leukemia by modification of the procedure of Kao et al. (1988). Briefly, granules were extracted by sonic disruption in 10 mM HCl. After centrifugation, the supernatant fluid was chromatographed on a Dymaxtron Orange A-agarose dye affinity column. Enzymatically active fractions were pooled and further purified on Trasylol-Sepharose and by ion-exchange chromatography on SP-Sepharose. The human proteinase-3 isolated by this method had little or no contamination with neutrophil elastase. The material was homogeneous as evaluated by LC electrospray-mass spectrometry, N-terminal amino acid sequence determination and SDS-polyacrylamide gel electrophoresis (manuscript in preparation).

NE was purchased from Elastin Products (Owensville, MO) and Pseudomonas elastase from Nagase Biochemicals (Tokyo, Japan). Human and mouse macrophage metalloelastases were generous gifts of Dr. Steven Shapiro.

**Elastase inhibitors.** Compounds used to inhibit elastinolytic activity were EDTA (10 mM), 1,10-phenanthroline (10 mM), α1-PI (77 μM), SLPI (0.71 μM), CMK (0.1 mM) (Enzyme Systems Products, Livermore, CA) and L-658,758 (0.1 mM) (Merck Research Laboratories, Rahway, NJ). For inhibition of individual sol samples with CMK and L-658,758 (table 2), inhibitor concentration was 0.1 mM. In other experiments, sol was incubated at 37°C for 30 minutes with varying concentrations of L-658,758, then assayed for activity towards elastin or synthetic substrate (fig. 3).

**Elastase assays.** Elastinolytic activity was determined by measuring the degradation of 3H-elastin (table 1 and fig. 1). 3H-elastin was prepared by reductive alkylation of bovine neck ligament elastin (Elastin Products, Owensville, MO) as described by Gordon et al. (Gordon et al., 1976). For assay of activity of NE, PsE and pooled CF sputum sol with and without inhibitors, 2 μg of elastase was assayed in 200 μl of 0.1 M NaCl, 0.125 M MOPS, pH 7.5, containing 800 μg of 3H-elastin (1030 cpm/μg elastin). The reaction mixture was incubated for 24 h at 37°C and then centrifuged at 13,000 × g for 15 min to pellet undegraded elastin. Solubilized 3H-peptides in the supernatant were measured by liquid scintillation counting. For figures 1 and 2, 100% activity of pooled sol represents degradation of 230 μg of elastin in 24 h. Human and mouse macrophage metalloelastase were assayed in the same manner (fig. 1), except that 5 mM CaCl2 was incorporated into the assay buffer to provide optimal conditions for activity. For assays in the presence of inhibitor, elastase and inhibitor were incubated for 30 min at 37°C before the addition of 3H-elastin substrate.

For assays of individual sol samples (table 2), elastinolysis was measured similarly but in a larger volume and in a buffer of higher ionic strength. A 20-μl aliquot containing 330 μg of 3H-elastin (1377 cpm/μg elastin) in 1 M NaCl, 10 mM KH2PO4, 0.015% Tween-20, pH 7.4, was combined with sol samples diluted 200-fold in 1 M NaCl, 0.125 M KH2PO4, pH 7.4, in a final volume of 0.97 ml. The assay proceeded for 24 h and was analyzed as described above. Elastinolytic activity was converted to an equivalent NE concentration by comparison with a nonlinear standard curve generated with pure active site-titrated human NE.

NE activity was also measured using a specific substrate, AAPV-pNA. The change in Abs 405 nm of a reaction mixture contained in 200 μl of 0.1 M NaCl, 0.125 M KH2PO4, pH 7.4, and 1 mM substrate was measured kinetically at room temperature in a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA). Standard curves, which were linear with concentration, were generated using known amounts of active site-titrated pure human NE.

For assays of pooled sol or NE with increasing concentrations of L-658,758 (fig. 3), elastase was incubated with an equal volume of inhibitor for 30 min at 37°C and then was diluted and assayed as described above.

**Statistical analyses.** Statistical analyses were performed by one-way analysis of variance (figs. 1 and 2).

**Results**

From 17 inpatients with CF who were undergoing i.v. antibiotic therapy to combat an acute pulmonary exacerbation, we collected 35 sputum samples. Ranging in age from 15 to 36, all these patients had moderately advanced disease. All subjects were colonized with Pseudomonas aeruginosa. Sputum samples, which averaged 9 ml, were fractionated into gel and sol phases. Then 22 samples from nine patients were pooled to prepare pooled CF sputum sol; 13 samples from eight patients were studied individually.

Three potential sources of elastase in CF sputum are the neutrophil, the macrophage and Pseudomonas. We investigated the ability of L-658,758 to block proteolysis by elastases from these sources and to block elastases in pooled CF
sputum sol. The elastin-degrading activity of a 10 µg/ml solution of NE, proteinase-3, human and mouse macrophage metalloelastase and Pseudomonas elastase in the absence of any inhibitor was measured under assay conditions that approximated physiological ionic strength (table 1). The macrophage metalloelastases were assayed similarly to the other elastases, but at a lower concentration to conserve enzyme (2.2 µg/ml) and in a solution containing 5 mM CaCl₂, because these enzymes are known to require millimolar Ca²⁺ for activity. All the purified elastases demonstrated considerable elastin-degrading activity, ranging from 20.3 µg elastin degraded/mg elastase/min to 218.3 µg elastin degraded/mg elastase/min. For this calculation, the elastase concentration in pooled CF sol was taken to be the NE concentration measured by hydrolysis of AAPV-pNA.

The activity of the elastases and pooled sol when combined with L-658,758 was then measured (fig. 1). L-658,758 was found to be an effective inhibitor of NE, proteinase-3 and sol elastase, but not of the human or mouse metalloelastase or of Pseudomonas elastase. We next measured the activity of a panel of inhibitors to block the elastinolytic activity of CF sputum sol (fig. 2). EDTA and 1,10 phenanthroline, which are potent inhibitors of human and mouse metalloelastase and of Pseudomonas elastase (Shapiro, 1994), had no significant inhibitory effect on CF sputum sol elastase. However, α₁-PI, SLPI, CMK and L-658,758 were potent inhibitors of sol elastase (Salveson and Travis, 1989; Llewellyn-Jones et al., 1994). Because all four inhibitors act against NE, and because SLPI does not inhibit proteinase-3 (Rao et al., 1993), these results suggest that virtually all the measurable elastinolytic activity for the group of samples was 10.52 g elastin/ml sol/min, and the range was 3.2 g elastin/ml sol/min for the sample with lowest activity to 26.3 g elastin/ml sol/min for that with the highest activity. Standard curves generated with pure NE show that these levels of elastinolysis are equivalent to micromolar levels of NE, ranging from 0.47 µM to 18.5 µM (table 2).

Elastinolytic activity could be measured in the sol phase of all of the 13 sputum samples studied individually (table 2). We conducted these assays at high ionic strength to maximize detection of any low levels of elastase. The mean activity for the group of samples was 10.52 µg elastin degraded/ml sol/min, and the range was 3.2 µg elastin/ml sol/min for the sample with lowest activity to 26.3 µg elastin/ml sol/min for that with the highest activity. Standard curves generated with pure NE show that these levels of elastinolysis are equivalent to micromolar levels of NE, ranging from 0.47 µM to 18.5 µM (table 2).

We evaluated the activity of the cephalosporin-based inhibitor L-658,758 and CMK to block the elastinolytic activity of the individual sols. In the presence of 0.1 mM CMK, sol elastin-degrading activity was reduced by more than 95% for all samples examined (table 2). Preincubation of sol with 0.1 mM L-658,758 blocked elastinolysis by more than 97% for all of the samples (table 2), a result that demonstrates the utility of this new inhibitor in blocking elastase activity in human airway secretions.

When two different sol preparations diluted to concentrations of 0.009 µM and 0.053 µM, respectively, were assayed in increasing concentrations of L-658,758, virtually all the elastinolytic activity could again be inhibited (fig. 3). In addition, the sol preparation that contained higher NE concentration required larger amounts of inhibitor to achieve half-maximal inhibition, which is consistent with titration of the enzyme activity by L-658,758. Inhibition of activity against the specific NE substrate AAPV-pNA was also demonstrated for sol containing 0.20 µM and 1.60 µM NE (fig. 3). L-658,758 was a potent antielastase, requiring only a few molar equivalents to inhibit 50% of CF sol elastase activity. Activity was inhibited 50% by 5.6, 1.9, 13.4 and 2.6 molar equivalents of inhibitor for sol NE concentrations of 0.009 µM, 0.053 µM, 0.20 µM and 1.60 µM, respectively.

### Discussion

This study demonstrates that the cephalosporin-based compound L-658,758 is an effective inhibitor of the purified elastases NE and proteinase-3, as well as of the elastinolytic activity of CF sputum. Human and mouse macrophage metalloelastase and Pseudomonas elastase were not inhibited by L-658,758. Sputum from CF patients was found to contain high levels of elastinolytic activity. This activity could in
large part be attributed to neutrophil elastase, because it was blocked by inhibitors of NE (α1-PI, SLPI, CMK, and L-658,758) but not by inhibitors of metalloelastases.

A number of studies have addressed the question of which types of elastases are present in CF airway secretions. Although some have suggested the presence of modest amounts of Pseudomonas elastase (Fick et al., 1984; Bruce et al., 1985), most have concluded that NE is the predominant elastase in CF airway secretions (Jackson et al., 1984; Suter et al., 1984; Tournier et al., 1985). The results of the present study are in keeping with these. However, the observation that a low level of sol elastase activity persisted in the presence of SLPI raises the possibility that a trace of active proteinase-3 may be present in CF sol.

**TABLE 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Elasin Degraded (μg/ml sol/min)</th>
<th>NE (μM)</th>
<th>Elasin Degraded (% of total) with CMK</th>
<th>Elasin Degraded (% of total) with L-658,758</th>
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<tr>
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<td>3.0</td>
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</tr>
<tr>
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</tr>
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<tr>
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<td>1.3</td>
</tr>
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</tr>
<tr>
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<td>18.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>H</td>
<td>13</td>
<td>4.2</td>
<td>1.20</td>
<td>0.0</td>
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</tbody>
</table>

* Elasinolytic activity was measured with 3H-elasin substrate in 1.0 M NaCl, 0.075% Tween 20, 0.125 M KH2PO4, pH 7.4.  
** Elasinolytic activity was converted to an equivalent NE concentration by comparison with a standard curve generated with human NE and reflects the assumption that all sol elastinolytic activity derived from NE.

**Fig. 2.** Inhibition of CF sputum sol elastase activity. The elastinolytic activity of pooled CF sputum sol was measured in the presence and absence of a panel of inhibitors. CF sputum sol was pretreated with inhibitors or assay buffer for 30 min at 37°C and then assayed for activity as described in the legend to figure 1. The inhibitors used were EDTA (10 mM), 1,10 phenanthroline (10 mM), α1-PI (77 μM), SLPI (0.71 μM), CMK (0.1 mM) and L-658,758 (0.1 mM). Activity for each individual elastase in the absence of inhibitor was taken to be 100%. Statistical analyses were performed by one-way analysis of variance; * indicates P < 0.05. Values are mean ± S.D. for assays performed two times in triplicate.

**Fig. 3.** Inhibition of CF sol elastase activity. Individual sol samples 12 and 13 (see table 2) were diluted to different concentrations of NE as shown. The sol was then incubated with increasing concentrations of inhibitor. NE activity was measured against elastin (closed figures) or AAPV-pNA (open figures). Sol sample 18, squares; sol sample 19, circles. Data were fitted to a sigmoid curve.

As predicted from their mechanisms of action, none of the antielastases with activity toward NE and proteinase-3 inhibited the metalloelastases, human and mouse metalloelastase and Pseudomonas elastase. Therefore, treatment with currently available NE inhibitors will not block proteolysis by human macrophage metalloelastase. Human macrophage metalloelastase is a potent protease that, although it does not appear in measurable quantities in CF sputum, may yet contribute to lung destruction in diseases such as CF and emphysema by acting locally at sites of macrophage binding.

The high levels of active NE in CF sputum sol demonstrate that important antiprotease defenses have been overwhelmed in the CF patient's airways and highlight the potential for extensive lung damage through proteolysis. Elevated elastinolytic activity in CF airway secretions can begin early in a patient's life (Birrer et al., 1994; Khan et al., 1995) and parallels the severity of lung disease (Suter et al., 1984; O'Connor et al., 1993). In a direct demonstration of the toxicity of elastase in CF airway secretions, CF sputum sol was shown to induce lung injury and inflammation when introduced into the lungs of healthy rats by intratracheal instillation (Rees and Brain, 1995). The hemorrhagic component of the observed injury was prevented by p.o. pretreatment of the rats with antielastase. These observations, combined with our knowledge of the variety of soluble and insoluble substrates that NE and proteinase-3 have been shown to degrade in vitro and of the injury they have been demonstrated to induce in animal models in vivo, all argue that reduction of elastase activity in the CF patient's lungs may prove beneficial (Bieth, 1986; Kao et al., 1988; Lucey et al., 1988; Rao et al., 1991).

Therefore, we further examined the ability of L-658,758 to reduce elastase activity in individual samples of CF sol. L-658,758 inhibited CF sol elastolysis virtually completely, with less than 1% of activity remaining in most samples. The concentration of L-658,758 required to achieve 50% inhibition varied with the concentration of NE present in the reaction mixture. This is consistent with its reported mech-
anism as a time-dependent, irreversible inhibitor that ti-
trates the active site of the enzyme. Approximately 5-fold
molar equivalents of inhibitor were sufficient to achieve 50%
inhibition, a result that demonstrates relatively little reaction
with water or other components of the reaction mixture.

The low concentrations of inhibitor required to achieve
virtually complete inhibition of elastinolytic activity of CF
sol or degranulating neutrophils in the reverse passive
arthus reaction (Fletcher et al., 1990a; Fletcher et al., 1990b; Rees and Brain, 1995). Other antielastases have also been shown to mitigate the toxic effects of NE. For example, in-
stilled α1-PI has been shown to reduce the development of
emphysma in hamsters exposed to NE (Stone et al., 1990).

These studies have demonstrated that a specific cephalos-
porin-based inhibitor of NE effectively blocks the elastino-
lytic activity of CF airway secretions in vitro. This raises the
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possibility that such inhibitors might be useful in controlling
excessive NE activity in the lungs of CF patients.


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